Metastatic Cells Can Escape the Proapoptotic Effects of TNF-α through Increased Autocrine IL-6/STAT3 Signaling

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Abstract

The liver is a common site for cancer metastases in which the entrance of tumor cells has been shown to trigger a rapid inflammatory response. In considering how an inflammatory response may affect metastatic colonization in this setting, we hypothesized that tumor cells may acquire resistance to the proapoptotic and tumoricidal effects of TNF-α, a cytokine that is elevated in a proinflammatory tissue microenvironment. In this study, we investigated molecular mechanisms by which such resistance may emerge using tumor cells in which the overexpression of the type I insulin-like growth factor receptor (IGF-IR) enhanced the inflammatory and metastatic capacities of poorly metastatic cells in the liver. Mechanistic investigations in vitro revealed that IGF-IR overexpression increased cell survival in the presence of high levels of TNF-α, in a manner associated with increased autocrine production of interleukin-6 (IL)-6. In turn, tumor cell–derived IL-6 induced gp130 and IL-6R–dependent activation of STAT3, leading to reduced caspase-3 activation and apoptosis. We found that IL-6 production and cell death resistance were dose dependent with increasing TNF-α levels. In addition, RNA interference–mediated knockdown of either IL-6 or gp130 that established a blockade to autocrine STAT3 induction was sufficient to abolish the prosurvival effect of TNF-α and to inhibit liver metastasis. Taken together, our findings define an IGF-IR–mediated mechanism of cancer cell survival that is critical for metastatic colonization of the liver. Cancer Res; 72(4); 865–75. ©2011 AACR.

Introduction

Cancer metastasis remains the main cause of cancer-related mortality. To colonize a distant site, cancer cells must complete several rate limiting steps, including detachment from the primary site and invasion, extravasation, survival in the circulation, extravasation, and finally, survival and growth in the target organ (1–3). Once in the target organ, cancer cells can induce a proinflammatory microenvironment involving host leukocytes and monocytes that reside in the affected tissue or are recruited in response to signals elaborated by the tumor cells. These host cells can further promote the development of secondary tumors by releasing cytokines and chemokines such as TNF-α, interleukin (IL)-1 and IL-6 that promote tumor cell migration, tumor survival, and the induction of endothelial cell adhesion receptors that mediate tumor cell arrest and transendothelial migration (4–6).

TNF-α is a pleiotropic, proinflammatory cytokine that can have multiple and diametrically opposing effects on tumor cell survival and malignant progression. Although at high concentrations, it can cause hemorrhagic necrosis, induce apoptosis, and stimulate antitumor immunity, it can also, under the appropriate conditions, promote tumor initiation and progression (7, 8). TNF-α is produced as a cell membrane–associated 26-kDa proprotein that can be cleaved by matrix metalloproteinases (MMP) to release a soluble 17-kDa form (reviewed in ref. 9). TNF-α is active as a self-assembling, noncovalently associated trimer that binds to 2 cell surface receptors; the p55/p60 TNFR1 and the p75/p80 TNFR2. Binding to TNFR1 triggers the recruitment of the adaptor TNFR-associated death domain that serves as a platform to assemble alternative signaling complexes. This can lead to activation of IKK/IκBα/NF-κB signaling and upregulation of NF-κB target genes such as IL-6 or the recruitment of Fas-associated death domain-containing protein and caspase 8, leading to activation and cleavage of the effector caspase-3 and apoptosis (10, 11).

IL-6 belongs to a family of cytokines that signal through receptor complexes containing the signal-transducing protein gp130 (12). It can play diverse roles both as a regulator of the acute inflammatory response and as a growth and survival factor. Binding of IL-6 to its cellular receptor IL-6Rα (CD126) triggers the recruitment of gp130 (CD130) and results in Janus-activated kinase (JAK) activation and phosphorylation of transcription factors of the signal transducer and activator of transcription (STAT) family, particularly STAT3. Phosphorylated STAT3 dimers translocate to the nucleus in which direct DNA binding leads to transcriptional activation of various
genes, including oncogenes such as c-Myc, cell survival genes such as Bcl-xl, cell-cycle regulators such as Ccnd1 (Cyclin D1), cytokines such as Il6, and molecular modulators of angiogenesis and metastasis such as Mmp2, Mmp9, Hif1α, and Vegf (13, 14). Activation of an autocrine regulatory loop whereby IL-6 activates STAT3 and STAT3 in turn, upregulates Il6 transcription was shown to maintain cell resistance to apoptosis and contribute to tumor progression (15). IL-6 can also bind to gp130 via an extracellular soluble form of IL-6Rα (sIL-6R) resulting in activation of gp130/JAK/STAT3 signaling and enhancement of the effects of IL-6 (16).

The receptor for the type I insulin-like growth factor (IGF-IR) has also been identified as a survival factor for cancer cells and implicated in cellular transformation, malignant progression, and the acquisition of an invasive/metastatic phenotype (17). Ligand-induced activation of the intrinsic IGF-IR tyrosine kinase can initiate several invasive/metastatic pathways including the Ras/Raf/ERK (ERK, extracellular signal-regulated kinase) pathway implicated in receptor-mediated mitogenesis and transformation and the phosphoinositide 3-kinase (PI3K)/Akt pathway implicated in the transmission of cell survival signals. Cross-talk with integrins and with the JAK/STAT pathway has also been described (17–19). Uregulated expression of IGF-IR has been documented in many types of cancers, and it has been recognized as a therapeutic target in the treatment of malignant disease (17, 20, 21).

Previously, we have shown that lung carcinoma M-27 cells ectopically expressing human IGF-IR acquired a liver-metastasizing potential that correlated with increased cell survival in the liver (22). We have also shown that metastatic lung and colon carcinoma cells that invade the liver initiate a rapid host inflammatory response that entails increased production of cytokines TNF-α and IL-1 by activated Kupffer cells and results in increased tumor cell adhesion to local microvascular endothelial cells, transmigration, and metastasis. This ability to induce a rapid inflammatory response was tumor type specific, correlated with the liver-metastasizing potential of the cells, and was also noted in cells with ectopic IGF-IR expression (6, 23, 24). These results imply that metastatic tumor cells acquire mechanisms of resistance to the proapoptotic effects of TNF-α produced in the microenvironment of the target organs to survive and give rise to new metastatic foci.

On the basis of these findings, we hypothesized that IGF-IR overexpression plays a role in the acquisition of resistance to the proapoptotic effects of TNF-α by shifting TNFRI-induced signals from pro- to antiapoptotic. The objective of this study was therefore to identify molecular mechanism(s) that contribute to the acquisition of TNF-α resistance in highly metastatic tumor cells and investigate the role that IGF-IR plays in this process.

Materials and Methods

Cells

Sublines M-27 and M-27IGFIR were generated in our laboratory. Their origin, properties, metastatic phenotypes, and culture conditions have been described in detail previously (22, 25). Murine colon carcinoma MC-38 cells that are highly metastatic to the liver in an IGF-I–dependent manner (26) were a kind gift from Dr. Shoshana Yakar (Mount Sinai Hospital, NY). These cell lines have been tested and authenticated as per the McGill University Animal Care committee and the McGill University Biohazard committee guidelines.

Antibodies and reagents

The polyclonal rabbit antibodies to p65, gp130, IL-6Rα (M-20), USF-2, and STAT3 (C-20) were from Santa Cruz Biotechnology. The monoclonal antibodies to Akt, p-Akt (Ser473), and p-STAT3 (D3A7, Tyr705) were from Cell Signaling Technology. The monoclonal mouse anti–β-actin antibody was from Sigma Chemical Co. The anti-rat Cathepsin B antibody was a generous gift from Dr. John S Mort (The Shriner's Hospital for children, Montreal, Quebec, Canada). The antibody to caspase-3 (#9662; Cell Signaling Technology) was a generous gift from Dr. Teruko Takeko (Department of Urology, McGill University, Quebec, Canada). The anti-murine IL-6 antibody (MAB406), a generous gift from Centocor Co., the goat antibody to mouse IL-6 Rα, and normal rat IgG1 were from R&D. The recombinant human IGF-I was from United States Biological. The recombinant TNF-α and goat serum immunoglobulin G (IgG) were from Invitrogen. The inhibitors JSH-23, LY294002, and PD98059 were from Calbiochem, a brand of EMD Biosciences, Inc.

Reverse-transcriptase PCR

RNA was extracted with the TRIzol reagent (Life Technologies), and reverse transcriptase PCR (RT-PCR) was carried out with the M-MLV reverse transcriptase and Taq DNA polymerase (both from Invitrogen), as we previously described (27). Primer sequences are listed in Supplementary Table S1.

Quantitative real-time PCR

RNA extraction and reverse transcription were carried out as described above. Quantitative real-time PCR (qRT-PCR) was carried out essentially as we previously described (27).

Western blotting

Cell fractionation and nuclear extraction were carried out as described in detail elsewhere (28). Whole-cell lysate or subcellular fraction proteins were separated by polyacrylamide gel electrophoresis (PAGE) using 10% SDS gels, and Western blot analysis was conducted as we previously described (29). The primary antibody dilutions are shown in Supplementary Table S2, and the secondary antibodies were used at a dilution of 1:10,000.

Apoptosis assay

Apoptosis was measured by an ELISA kit for detection of mono-oligonucleosomes (Roche Diagnostics), as per the manufacturer’s instructions using 50,000 cells per analysis.

ELISA

Tumor cell–conditioned media were harvested, concentrated 100× with Centrifugal Filter Units (Milipore), and IL-6 concentrations quantified with the mouse IL-6 DuoSet ELISA Development System (R&D Systems).
Gene silencing by short hairpin RNA

Tumor cells cultured in 6-well plates (3 × 10^5 cells per well) were transfected with the pRS plasmid (Origene) expressing gp130 or IL-6 short hairpin RNA (shRNA) sequences or a scrambled sequence as a control, using serum-free media containing Lipofectamine. Puromycin (4 μg/mL) was added 48 hours later and drug-resistant cells cloned by the limiting dilution method. Clones with a stable reduction in the respective mRNA levels were selected for further study.

Experimental metastasis assay

Experimental liver metastases were generated by the intrasplenic/portal injections, as we previously described (22, 30). Metastases visible on the surface of the liver were enumerated on April 13, 2017. © 2012 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from

Results

Increased resistance to TNF-α–induced apoptosis in liver-metastasizing tumor cells

Previously, we have shown that highly metastatic lung and colon carcinoma cells that invade the liver initiate a rapid host inflammatory response that entails increased production of the cytokine TNF-α and results in increased tumor cell adhesion, transmigration, and metastasis (6, 23). This prompted us to investigate whether the ability of metastatic cells to survive in this local inflammatory microenvironment involved an altered response to TNF-α. To this end, we used Lewis lung carcinoma subline M-27 cells that acquired a liver-metastasizing potential following ectopic expression of the human IGF-I receptor (M-27^IGFR cells; refs. 22, 31). We compared the response of these, and wild type, poorly metastatic M-27 cells to treatment with increasing concentrations of TNF-α by measuring apoptosis with a detection system for mono-oligonucleosomes. We observed that M-27^IGFR cells had an altered response, particularly at high TNF-α concentrations. Although at the lower concentration of 10 ng/mL, TNF-α in serum-free medium could partially protect both cell types from apoptosis (50% reduction), at the higher concentrations of 25 to 50 ng/mL, M-27^IGFR, but not M-27 cells were significantly protected (> 85% reduction at 50 ng/mL) from cell death (Fig. 1A), suggesting that under these conditions, TNF-α activated distinct signaling pathways in these cells.

TNFR activation can lead to the induction of caspase-3–mediated cell apoptosis when death domain signaling predominate. We therefore analyzed caspase-3 expression and activation levels in these cells under basal conditions and in response to TNF-α. We found that the metastatic M-27^IGFR cells expressed reduced caspase-3 mRNA and protein levels relative to M-27 cells and these levels declined even further when the cells were treated with TNF-α (Fig. 1B and C). When the tumor cells were treated with TNF-α, an increase in cleaved caspase-3 mRNA and protein levels were observed in both cell types, although the increase was more pronounced in M-27 cells (Fig. 1B and C).

Figure 1. IGF-IR expression increases the antia apoptotic effect of TNF-α. A, cells in serum-free RPMI medium were treated (or not) with the indicated concentrations of TNF-α for 48 hours, lysed, and the cytoplasmic mono-oligonucleosomes analyzed by ELISA. The results are expressed as apoptosis (means and SD) relative to the respective control untreated cells, based on 3 separate experiments. B, constitutive caspase-3 expression levels were analyzed by qRT-PCR. Shown are means and SD of 3 separate analyses normalized to glyceraldehyde-3-phosphate dehydrogenase and relative to M-27 cells that were assigned a value of 1. C, cells were treated (or not) with 10 ng/mL TNF-α for 48 hours and caspase-3 protein levels in total cell lysates analyzed by Western blotting. Shown are representative results of 3 analyses conducted. D, cells were treated with 50 ng/mL TNF-α for the indicated time interval, lysed, and the intact and cleaved forms of caspase-3 detected by Western blotting after loading 200 μg total protein per lane. Shown are representative results of 2 analyses conducted. *, P < 0.05. **, P < 0.01.
caspase-3 levels could be detected by Western blotting in M-27, but not in M-27IGFIR cells (Fig. 1D), even when the protein load for the latter cells was doubled to adjust for the relative decrease in total caspase-3 levels (Supplementary Fig. S1). This difference in detectable cleaved caspase-3 levels was consistent with the reduction in apoptosis seen in M-27IGFIR cells.

Increased IL-6/STAT3 signaling in metastatic M-27IGFIR cells

Caspase-3 production is negatively regulated by activated STAT3. We therefore next analyzed the expression levels and activation status of STAT3 in these cells. qRT-PCR and Western blotting (Fig. 2A and B) revealed that basal STAT3 expression levels in M-27IGFIR cells were actually lower than those in nonmetastatic M-27 cells. However, activated STAT3 (p-STAT3) levels were markedly increased (4-fold) in M-27IGFIR cells (Fig. 2B). Furthermore, treatment of M-27IGFIR, but not M-27 cells with TNF-α for 24 hours increased p-STAT3 levels and a further increase of 10% to 50% was seen when IGF-I was also added (Fig. 2C and D). STAT3 activation was also observed when M-27Mock cells were stimulated with TNF-α (with or without IGF-I) for a shorter interval of 4 hours (Supplementary Fig. S2). This indicated that upon cell treatment with TNF-α, a signal transduction mechanism upstream of STAT3 was activated and suggested that in these cells, there was an increased production of a STAT3-activating cytokine under basal conditions that could be further augmented by TNF-α.

IL-6 is a potent activator of JAK/STAT signaling and in both M-27 and M-27IGFIR cells, treatment with exogenous IL-6 led to STAT3 activation (Supplementary Fig. S3). When we measured IL-6 mRNA expression levels and compared secreted levels in the conditioned media by ELISA, we found that basal IL-6 mRNA levels were 2- to 4-fold higher in M-27IGFIR cells, as shown in Fig. 3A and B, and this corresponded to an increase in protein production levels, as shown in Fig. 3D (extreme left bars). Furthermore, when IL-6 production was measured following cell treatment with TNF-α, a marked difference was observed in the response of these tumor cells. Namely, whereas in M-27 cells, IL-6 mRNA and protein levels remained virtually unchanged, in M-27IGFIR cells, they were highly induced, with mRNA levels increasing 7.5- to 9.5-fold, as measured by qRT-PCR (Fig. 3C) and protein production increasing up to 60-fold, as measured by ELISA (Fig. 3D). Similarly, in murine colorectal carcinoma MC-38 cells that are highly metastatic to the liver in an IGF-I-dependent manner (26), IL-6 production was markedly increased (60-fold) in response to treatment with TNF-α (Fig. 3E). Furthermore, in both cell types, the increase in IL-6 production was TNF-α dose dependent and could be further augmented when the cells were treated simultaneously with TNF-α and IGF-I (Fig. 3C–F), confirming that signals generated by ligand-activated IGF-IR could enhance transcriptional activation of IL-6. The TNF-α–inducible increase in IL-6 production was mediated mainly via NF-κB activation because pretreatment of M-27IGFIR cells with JSH23, a cell-permeable diamino compound that selectively blocks nuclear translocation of p65 (Rel-A; Supplementary Fig. S4), diminished the increase in IL-6 expression due to TNF-α in the presence or absence of IGF-I (Fig. 3G and H). In these experiments, IL-6 concentrations in the conditioned media of stimulated cells were within a range (17–252 pg/mL) that was confirmed to activate STAT3 in these cells (see, Supplementary Fig. S5).

The soluble IL-6 receptor α (sIL-6Rα) acts as an agonist to activate the gp130/JAK/STAT3 pathway. It was therefore of interest to analyze whether TNF-α treatment affected its
with Western blotting, we found that M-27IGFIR cells produced a higher level of the soluble receptor. However, this level did not suggesting that the effect of TNF-α in M-27IGFIR cells of IGF-I does not directly activate STAT3 signaling in these cells. Moreover, STAT3 activation in response to TNF-α was required to stimulate IL-6 production and autocrine STAT3 activation in these cells. However, the presence of TNF-α on STAT3 activation was not due to changes in the release of sIL-6R, although its presence per se may have contributed to enhanced STAT3 activation in these cells.

**IGF-I does not directly activate STAT3 signaling in M-27IGFIR cells**

The IGF-IR has previously been implicated in STAT3 signaling (32). To assess its role in STAT3 activation in our cells, M-27IGFIR cells were stimulated directly with IGF-I and the effect on STAT3 phosphorylation analyzed by Western blotting. Treatment of the cells with IGF-I for up to 2 hours failed to increase phospho-STAT3 levels (Fig. 4A) under conditions that triggered signaling, as evidenced by rapid Akt phosphorylation (Fig. 4B), suggesting that IGF-IR signaling could not directly activate STAT3 in these cells and that its major effect was to augment TNF-α/NF-κB signaling and thereby IL-6 production. Stimulation of the cells with IGF-I for 24 hours also failed to increase p-STAT3 levels (Supplementary Fig. S7), suggesting that the presence of TNF-α was required to stimulate IL-6 production and autocrine STAT3 activation in these cells. Moreover, STAT3 activation in response to TNF-α and IGF-I could be inhibited by a neutralizing antibody to IL-6Rα (Fig. 4C), providing direct evidence for the involvement of IL-6 in STAT3 activation by these ligands. To identify the signal transduction pathway downstream of IGF-IR involved in the enhancement of TNF-α/NF-κB–induced IL-6 transcription, we used the chemical inhibitors LY294002 and PD98059 to block PI3K and ERK signaling, respectively. Inhibition of PI3K signaling completely abolished the incremental increase in IL-6 production by the tumor cells. When conditioned media obtained from M-27 and M-27IGFIR cells before and after TNF-α treatment were analyzed for the presence of sIL-6R with Western blotting, we found that M-27IGFIR cells produced a higher level of the soluble receptor. However, this level did not increase following TNF-α treatment (Supplementary Fig. S6), suggesting that the effect of TNF-α on STAT3 activation was not due to changes in the release of sIL-6R, although its presence per se may have contributed to enhanced STAT3 activation in these cells.
production due to IGF-IR activation, while also reducing (2-fold) the effect of TNF-α alone. Inhibition of ERK signaling also reduced the increase in IL-6 production due to IGF-I, but to a lesser extent (64%; Fig. 4D). The results implicate both pathways in IGF-I-mediated enhancement of IL-6 synthesis, although PI3K/Akt signaling seems to play a more critical role.

Silencing of gp130 or IL-6 abolishes the antiapoptotic effect of TNF-α

The protein gp130 is an IL-6 co-receptor required for transmitting IL-6-derived signals. We confirmed that M-27IGFR cells express both gp130 and IL-6Rα (Supplementary Fig. S8A). To determine the role of gp130 in maintaining STAT3 activity and the prosurvival effect of TNF-α in these cells, we generated an M-27IGFR cell line with a stable knockdown of gp130 with shRNA (see Supplementary Fig. S8B and S8C). In these cells, the transcriptional activity of STAT3 was markedly reduced as shown by significant downregulation of the STAT3 target genes—Ih6 (>99% reduction) and Mmp2 (90% reduction) and a concomitant upregulation (10-fold increase) in the expression of Casp3 (Caspase-3) (Fig. 5A), confirming that gp130 signaling was essential for STAT3 activity in these cells. When the effect of stable knockdown of gp130 on the ability of TNF-α to rescue the tumor cells from apoptosis was analyzed, we found that a knockdown of gp130 expression significantly decreased the ability of TNF-α to rescue M-27IGFR cells from apoptosis and also reversed the antiapoptotic effect seen when the cells were cotreated with TNF-α and IGF-I (Fig. 5B). Similarly, tumor cells in which IL-6 production in response to TNF-α was blocked by shRNA-mediated silencing of IL-6 (Supplementary Fig. S9A and S9B), completely lost the survival advantage in the presence of high TNF-α concentrations (Fig. 5B) and could not be protected by the addition of IGF-I. Together, these results indicated that IL-6/gp130 signaling was essential for the prosurvival effect of TNF-α on these cells.

Loss of metastatic potential in cells with reduced autocrine IL-6-mediated survival signaling

Finally, to assess the effect of IL-6 inhibition on tumor cell potential to colonize the liver, we used M-27IGFR and MC-38 cells in which IL-6 expression was stably silenced by shRNA (Supplementary Figs. S9 and S10), as well as tumor cells treated with a neutralizing anti-IL-6 antibody prior to injection. As already shown above, the prosurvival effect of TNF-α (with or without IGF-I) was abolished in M-27IGFR cells stably transfected with IL-6 shRNA (Fig. 5B). Similarly, in MC-38 cells with silenced IL-6 expression that resulted in reduced STAT3 activation levels (Fig. 6A), TNF-α–mediated apoptosis levels were significantly increased (Fig. 6B). Moreover, when M-27IGFR cells were treated with a neutralizing antibody to murine IL-6 expression.
Survival. Our findings that TNF-α can rescue cells from cell death under conditions of serum deprivation may be particularly relevant to cell fate during the early stages of liver metastasis when the tumor cells may encounter the combined insult of ischemia/reperfusion due to sinusoidal vessel occlusion (33) and a surge in the local production of TNF-α, as we and others have previously shown (23; reviewed in ref. 34). Our results are in agreement with other studies that identified IL-6 as a survival factor in the liver both for primary hepatocellular carcinoma (35) and for liver metastases (36). However, whereas in those studies, IL-6 was liver derived and acting in a paracrine fashion, our results show that in the presence of inflammatory stimuli, tumor cells can activate this survival mechanism in an autocrine fashion and thereby escape the proapoptotic effects of the microenvironment. Of note, we did not observe a significant increase in circulating IL-6 levels (as measured by ELISA) in mice injected with M-27IGFIR or MC-38 cells with stable knockdown of IL-6, which suggests that IL-6 is not the key factor regulating this process in the context of tumor development.

Discussion

Our results show that in the presence of high TNF-α concentrations, highly metastatic tumor cells could be rescued from apoptosis by activating autocrine IL-6/gp130/STAT3 prosurvival signaling and implicate the IGF-IR axis in shifting the balance from cell death toward IL-6 induction and cell survival. We observed that at a lower concentration (10 ng/mL), TNF-α also had an antiapoptotic effect on M-27 cells that was lost at
of 0.5 observed that following treatment of both M-27 and M-27IGFIR other systems in which TNF-α could be shown to mediate a mechanism mediating the anti- and proapoptotic effects of TNF-α, p65/RelA protein is activated and translocates to the nucleus (unpublished observation), in which it can directly activate transcription of antiapoptotic factors. However, we did not observe a significant upregulation of IL-6 expression or increased STAT3 activation in M-27 cells at any of the TNF-α concentrations used.

In our M-27/M-27IGFIR model, the upregulation of IL-6 production in response to TNF-α depended on increased IGF-IR expression levels in the tumor cells. We observed that although ligand-induced activation of IGF-IR did not, in itself, significantly increase IL-6 production and STAT3 activation, the activated receptor could act synergistically with TNF-α to increase IL-6 levels. This effect was RelA/p65 dependent because it was inhibited when p65 translocation was blocked. It was also abolished when PI3K signaling was inhibited; suggesting that IGF-IR/Pi3K and NF-kB signaling converged to enhance IL-6 production in these cells. This synergistic effect was not unique to the M-27IGFIR cells as it was also observed in the liver-metastasizing Lewis lung carcinoma variant H-59 (results not shown) and murine colon carcinoma MC-38 cells (Fig. 3E) that express high IGF-IR levels and can also induce an acute inflammatory response in the liver (26, 38), suggesting that this survival mechanism is a broader phenomenon for liver-metastasizing cells.

Although the cross-talk mechanism(s) between the IGF and TNF-α axes in our cells remain(s) to be fully characterized, 2 potential interactions are likely candidates. Il6 gene expression is activated by transcription factors NF-κB, AP-1, and C/EBP, and IGF-IR was identified as an upstream activator of AP-1—dependent transcription (39, 40). It is possible, therefore, that the synergistic effect of IGF-IR and TNF-α is due to the concomitant activation of the 2 cis-acting transcription elements (NF-κB and AP-1) in the IL-6 promoter. Alternatively, IGF-IR may increase NF-kB signaling more directly, through the PI3K pathway. Indeed, TNF-α was identified as a direct activator of Akt phosphorylation in premalignant keratinocytes (41), and NF-kB was shown to be activated downstream of the PI3K/Akt pathway in prostate epithelial cells (42). This is consistent with our finding that the IGF-1 effect in our model was PI3K dependent and suggests that IGF-IR may act directly on the NF-kB pathway via PI3K/Akt signaling to enhance IL-6 production in response to TNF-α.

In this context, it is noteworthy that low levels of IL-6 and p-STAT3 could be detected in M-27IGFIR (but not M-27) cells under basal conditions, even without stimulation of the cells by exogenous IGF-I. This may be due to serum IGF-I and insulin present in the culture medium that maintain a basal level of IGF-IR/PI3K/Akt and ERK signaling in these cells, or to an autocrine effect of the low levels of IGF-II (but not IGF-1) produced by these cells (unpublished observation). Moreover, Igf2r expression in these cells was found to be reduced 2.5-fold relative to M-27 cells (unpublished gene array data). Because IGF-IR does not activate intracellular signaling and acts as a “sink” to reduce ligand bioavailability (43), a reduction in its levels in the presence of tumor-derived IGF-II could result in low level autocrine IGF-IR activation and signaling that are further enhanced in the presence of exogenous ligand.

Figure 6. Loss of survival advantage in cells with reduced IL-6 expression or function. A, STAT3 activation was measured in nontransfected MC-38 cells (WT) and cells stably expressing IL-6 shRNA (IL-6sh) or a scrambled sequence (Ctl; see Supplementary Fig. S8). All cells were treated (or not) with 50 ng/mL TNF-α for 24 hours and Western blotting carried out on total cell lysates. B, apoptosis was measured following treatment of the above cells with serum-free RPMI containing (or not) 50 ng/mL TNF-α. Cells (10⁵ per condition) were lysed and cytoplasmic mono-oligonucleosomes were analyzed with the Cell Death detection ELISA Kit (Roche). Results shown are based on a representative experiment carried out in triplicates. They are expressed as mean optimum density (O.D.) values (405 nm) relative to the respective untreated cells that were assigned a value of 1. M-27IGFIR, cells in C were treated with an anti-mouse IL-6 antibody or normal rat IgG1 as control, both at a concentration of 0.5 μg/mL and then with 50 ng/mL TNF-α. Apoptosis was measured as in B and the results, based on 3 separate experiments, are expressed relative to cells treated with TNF-α only. †, P < 0.05; ‡, P < 0.01 for the comparison groups indicated.

higher concentrations. This is consistent with observations in other systems in which TNF-α could be shown to mediate opposing effects at different concentrations (7). Although the mechanism mediating the anti- and proapoptotic effects of TNF-α in these cells remain to be fully elucidated, we have observed that following treatment of both M-27 and M-27IGFIR cells with 10 ng/mL TNF-α, the NF-kB p65/RelA protein is activated and translocates to the nucleus (unpublished observation), in which it can directly activate transcription of antiapoptotic factors. However, we did not observe a significant upregulation of IL-6 expression or increased STAT3 activation in M-27 cells at any of the TNF-α concentrations used.

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Autocrine IL-6/STAT3 signaling in these cells may also be enhanced by increased production of sIL-6R that acts as an agonist to activate gp130. Although the regulatory link between increased IGF-IR expression and the increased production of sIL-6R in our cells is presently unknown, we have previously shown that M-27 and M-27IGFIR cells express distinct MMP repertoires (27, 29, 44). It is possible therefore, that cleavage of the membrane-bound IL-6R by a proteinase preferentially expressed or activated in M-27 IGFIR cells is involved. Our data indicate however, that TNF-α did not directly regulate sIL-6R production levels, suggesting that its major role in STAT3 activation was mediated through the upregulation of IL-6.

Cross-talk between the IGF axis and the IL-6/STAT3 survival pathway has been documented in other models. The evidence for this association is particularly strong for multiple myeloma cells in which cosignaling by the IGF-IR and IL-6R were shown to be critical for survival and growth (45, 46). Conversely in T cells, the neutralization of IL-6 could partially abrogate the antiapoptotic effect of IGF-I (47), suggesting that IGF-IR and IL-6 signaling were interdependent in these cells. To our knowledge, however, our results are the first to document a synergistic effect of IGF-IR signaling in the induction of autocrine IL-6/STAT3 signaling by TNF-α. Moreover, the data suggest that the ability of the IGF axis to regulate IL-6 production and STAT3 signaling may be a function of the NF-κB activation status in the cells and therefore provide insight into the cross-talk between the IGF axis and NF-κB signaling.

In some studies, IGF-IR was identified as a direct activator of JAK/STAT signaling (48). However, STAT3 activation was not

Figure 7. Gene silencing or blockade of IL-6 reduce the liver-metastasizing potential of tumor cells. Liver metastases were enumerated 20 days after the intrasplenic/portal inoculation of 2 x 10^6 M-27IGFIR cells (A and B) or 5 x 10^5 MC-38 cells (C–E) stably expressing IL-6 shRNA (IL-6sh) or mock-transfected (Ctl). F, in a separate experiment, M-27IGFIR were pretreated for 1 hour with 50 μg/mL of an anti-mIL-6 antibody, or vehicle as control, prior to the injection. Metastases were enumerated (and sized) before fixation of the liver and the livers then fixed in 10% neutral buffered formalin for the preparation of paraffin blocks. Shown are the numbers of metastases in individual livers and means (horizontal bar) per group in a representative experiment of 2 carried out with M-27IGFIR (A, B, and F) and 2 with 2 different clonal sublines of MC-38 with IL-6 silencing (C–E). Numbers on the top indicate the incidence of hepatic metastases per group and below the mean nodule size. Representative livers from one of these experiments are shown in B and D, and representative hematoxylin and eosin-stained sections of formalin-fixed, paraffin-embedded livers from an experiment with MC-38 cells showing (top) a metastasis corresponding to the mean nodule size in this group are shown in E. Magnification in E: images on left, 50 μm; image in inset on right (same metastases as shown on the left), 400 μm. **, P < 0.05; ***, P < 0.01 in comparison with control group as determined by the Mann-Whitney test. L, liver; T, tumor.
observed in M-27IGFR cells stimulated with IGF-I for up to 24 hours and was blocked in the presence of an antibody to IL-6R, while in MC-38 cells, the silencing of IL-6 reduced p-STAT3 levels. These data suggest that in the present cells, STAT3 activation was IL-6 dependent.

The receptor gp130 is an essential component of the IL-6 receptor complex and is required for the activation of JAK/STAT3 signaling (49). We show here that stable silencing of gp130 or IL-6 in different metastatic tumor cells abolished the antiapoptotic effect of TNF-α and that inhibition of IL-6 blocked hepatic metastasis. These data are further evidence that the prosurvival effect of TNF-α depended on IL-6/gp130/STAT3 signaling in these cells. In this respect, our findings are in line with other studies that identified IL-6 and STAT3 as progression factors and potential prognostic predictors and therapeutic targets in several malignancies, including hepatocellular (50), colon (51), pancreatic (52), and gastric (53) carcinomas that either arise in, or are commonly metastatic to the liver.

Among the genes that are transcriptionally regulated by STAT3 are mediators of cell death and survival (e.g., Casp3 and BclXL), cell-cycle progression factors (CcnD1), cytokines and chemokines (e.g., Il6, Cxcl5), and mediators of migration (Ctn), invasion (Mmp2, Mmp9), and angiogenesis (Hif1α, Vegf, ref. 54) that can all contribute to tumor progression and the cross-talk between inflammation and carcinogenesis. We observed that inhibition of STAT3 phosphorylation by gp130 silencing significantly decreased Il6 and Mmp2 gene expression while also increasing Casp3 expression levels. MMP-2 was identified as a critical mediator of liver metastasis (55), and we have previously shown that Mmp2 is an IGF-IR/PI3K–regulated gene in M-27IGFR cells (44, 56). The present results suggest that the constitutive increase in IL-6/STAT3 activity in these cells (even in the absence of TNF-α stimulation) may also contribute to the increased expression of MMP-2. In addition, the increase in Casp3 expression in gp130-silenced cells confirms that the prosurvival effects seen in the presence of TNF-α were indeed linked to the STAT3-mediated reduction in caspase-3 levels.

Collectively, these results provide new insight into the multifaceted role that the IGF axis can play in providing survival and growth cues to cancer cells in the proinflammatory microenvironment of the liver. They also identify additional candidate molecules in the search for antimetastatic, therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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